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(54) Title: MICROBIAL 11α-HYDROXYLATION OF STEROIDS

(57) Abstract

The invention relates to a microbial method of in vitro transformation of a steroid into its corresponding 11α-hydroxy analogue using oxygen and a microorganism selected from *Aspergillus ochraceus, Aspergillus niger, Rhizopus stolonifer, Rhizopus nigricans, Rhizopus arrhizus*, and strains of *Pestelotia*, characterized in that a steroid having a purity of less than 97 % is used.

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MICROBIAL 11α-HYDROXYLATION OF STEROIDS

The present invention relates to a microbial method of 11α-hydroxylation of steroids.

Microbial 11α-hydroxylation of steroids is a well known process, in vivo as well as in vitro. For instance 11α-hydroxylation of progesterone by cell-free preparations of *Aspergillus ochraceus* has been reported by Shibahara et al., Biochim. Biophys. Acta, 202 (1970), 172-179. It has also been known that microbial 11α-hydroxylation reactions of steroids are unpredictable, and invariably lead to incomplete transformations. Typically conversion degrees of 80-85% are obtained. For industrial applications it is however of importance to obtain high predictable conversion rates, which preferably lead to higher than 95% yields of 11α-hydroxylated steroids. Mathematical models in the optimization of such fermentation processes are discussed by Deshayes et al., Bull. Soc. Chim. Fr., (1980), II 24-34. For example, in the 11α-hydroxylation of canrenone, Deshayes disclosed that under optimum conditions better than 95% yields could be attained when *Aspergillus ochraceus* was used in a medium containing i.a. 10 g/l of glucose in a matrix of malt-extract and trypticase. Under these conditions up to 1.5 g/l of canrenone could be transformed, which was considered to be an improvement in the art, for instance as disclosed by Blunt et al., J. Chem. Soc., 6 (1971), 1136, who were not able to obtain more than 90% yield of 11α-hydroxylated canrenone using at the most 0.5 g/l of substrate.

Since it can be reasoned that contaminations will have a detrimental influence on the microbial process, it can be expected that further optimization could be obtained by increasing the purity of the starting materials. Surprisingly however, it has now been found that a further improvement is obtained by using less than pure substrate, in particular by using a substrate having a purity of less than 97%. The invention therefore relates to a microbial method of in vitro transformation of a steroid into its corresponding 11α-hydroxy analogue using oxygen and a micro-organism selected from *Aspergillus ochraceus*, *Aspergillus niger*, *Rhizopus stolonifer*, *Rhizopus nigricans*, *Rhizopus arrhizus*, and strains of *Pestelotia*, characterized in that a steroid having a purity of less than 97% is used.

Preferably the micro-organism is *Aspergillus ochraceus*. The purity of the steroid is preferably more than 90%. More preferably the purity of the steroid is between 90 and 95 %. The present method using impure substrates affords microbial transformations at substrate concentrations, which are substantially greater than the maximum concentrations as disclosed by Blunt or Deshayes.

The present microbial method can be used with steroids having an unsubstituted 11-position. Preferred examples are estr-4-ene-3,17-dione and canrenone.

The surprising effect of impurities on the conversion degree is illustrated in the following tables:

Table I

Conversion of estr-4-ene-3,17-dione by *A. ochraceus*		
purity (%)	substrate concentration (g/l)	conversion degree (%)
99	15	78
99	10	83
98	25	85
94*	15	91
94*	15	91
94*	15	94
93	25	97
92	10	98

* various natural impurities added to pure substrate

Table II

Conversion of canrenone by *A. ochraceus*		
purity (%)	substrate concentration (g/l)	conversion degree (%)
100	5	74
100	10	78
100	20	73
100	35	72
96	10	98
94	15	96
96	22	96
95	22	95

The invention is further illustrated by the following examples.

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EXAMPLES

Example 1

A shake flask containing a mineral growth medium with glucose was inoculated with spores of *A. ochraceus* and placed on a reciprocal shaker at 28 °C for 15 h. A stirred fermentor containing 5 l of medium was subsequently inoculated with 250 ml of germinated spore suspension. The used medium contained a glucose/yeast extract medium (glucose 40 g/l, yeast extract 10 g/l, pH 5.0). The culture conditions were as follows: stirrer speed 750 rpm, airflow 0.2 l/l/m, temp 28 °C. Foaming was measured with an antifoam electrode and controlled by automatic addition of a silicon based antifoam agent. When the culture had a biomass concentration of at least 2 g/l a small portion of estr-4-ene-3,17-dione (1 g/l) was added to induce the synthesis of the hydroxylation enzymes. Three hours later higher concentrations of steroid were added to reach the desired concentrations as given in Table I. The steroid transformation was stopped when repeatedly no increase in conversion was observed.

Example 2

In a shake flask the same culture as described in Example 1 was prepared. When the culture had a biomass concentration of at least 4 g/l the canrenone was added at once to reach the desired concentrations as given in Table II. The steroid transformation was stopped when repeatedly no increase in conversion was observed.

CLAIMS

1. A microbial method of in vitro transformation of a steroid into its corresponding 11α-hydroxy analogue using oxygen and a micro-organism selected from *Aspergillus ochraceus*, *Aspergillus niger*, *Rhizopus stolonifer*, *Rhizopus nigricans*, *Rhizopus arrhizus*, and strains of *Pestelotia*, characterized in that a steroid having a purity of less than 97% is used.

2. The method according to claim 1, wherein the micro-organism is *Aspergillus ochraceus*.

3. The method according to claim 1 or 2, wherein the purity of the steroid is between 90 and 95%.

4. The method according to any one of claims 1-3, wherein a steroid concentration greater than 0.5 g/l is used.

5. The method according to any one of claims 1-4, wherein the steroid is selected from estr-4-ene-3,17-dione and canrenone.

INTERNATIONAL SEARCH REPORT

Interr ·al Application No
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A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12P33/10

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C12P

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	US 3 294 646 A (SMITH, LELAND LEROY ET AL) 27 December 1966 see claims ---	1,2,5
Y	BULL. SOC. CHIM. FR. (1980), (1-2, PT. 2), 24-34 CODEN: BSCFAS;ISSN: 0037-8968, 1980, XP002029426 DESHAYES, CHRISTIAN M. P.: "Use of mathematical models for optimization in fermentation. Applications to transformations by microorganisms" cited in the application see page 31 --- -/--	1,2,5

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

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C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	J. STEROID BIOCHEM. (1970), 1(3), 221-7 CODEN: JSTBBK, 1970, XP000671122 TAN, LIAT ET AL: "Interactions of steroids and fungi. III. 11.alpha.-Hydroxylation and degradation of progesterone-4-14C by a cell-free preparation from Aspergillus ochraceus" see the whole document	1
Y	FR 1 091 743 A (PFIZER) 14 April 1955 see claims	1
Y	FR 1 406 102 A (UPJOHN) 24 November 1965 see claims	1
Y	FR 1 555 991 A (UPJOHN) 31 January 1969 see claims	1
Y	FR 1 261 181 A (MERCK) 8 September 1961 see claims; examples	1
Y	FR 1 080 205 A (UPJOHN) 7 December 1954 see claims; table IV	1
Y	NL 6 409 039 A (MERCK) 7 February 1966 see claims	1

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INTERNATIONAL SEARCH REPORT

.ormation on patent family members

Intern ıal Application No
PCT/EP 96/05729

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
US 3294646 A	27-12-66	NONE	
FR 1091743 A	14-04-55	NONE	
FR 1406102 A	24-11-65	BE 649398 A	
		CH 460759 A	
		DE 1240522 B	
		GB 1006818 A	
		NL 6406865 A	18-12-64
		US 3201324 A	17-08-65
FR 1555991 A	31-01-69	BE 711016 A	20-08-68
		CH 491893 A	15-06-70
		DE 1668671 A	23-09-71
		FR 7887 M	04-05-70
		NL 6801762 A	21-08-68
FR 1261181 A	08-09-61	NONE	
FR 1080205 A	07-12-54	NONE	
NL 6409039 A	07-02-66	NONE	